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Zhentao Sheng

University of Kentucky, zhentao.sheng1@uky.edu

Jingjing Xu

University of Kentucky, jingjing.xu@uky.edu

Hua Bai

University of Kentucky, hua.bai@uky.edu

Fang Zhu

University of Kentucky, fangzhudy@uky.edu

Subba R. Palli

University of Kentucky, rpalli@uky.edu

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Juvenile Hormone Regulates Vitellogenin Gene Expression through Insulin-like Peptide Signaling Pathway in the Red Flour Beetle, *Tribolium castaneum**

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Zhentao Sheng, Jingjing Xu, Hua Bai, Fang Zhu, and Subba R. Palli¹

From the Department of Entomology, College of Agriculture, University of Kentucky, Lexington, Kentucky 40546

Background: Juvenile hormone (JH) and nutrition signals regulate vitellogenin (Vg) synthesis in insects.

Results: Expression of genes coding for insulin-like peptides, insulin receptor, Akt, and FOXO is required for transduction of JH and nutrition signals.

Conclusion: JH and nutritional signals function through insulin-like peptide signaling pathway.

Significance: These studies illustrate the complex nature of endocrine regulation of reproduction.

Our recent studies identified juvenile hormone (JH) and nutrition as the two key signals that regulate vitellogenin (Vg) gene expression in the red flour beetle, *Tribolium castaneum*. Juvenile hormone regulation of Vg synthesis has been known for a long time in several insects, but the mechanism of JH action is not known. Experiments were conducted to determine the mechanism of action of these two signals in regulation of Vg gene expression. Injection of bovine insulin or FOXO double-stranded RNA into the previtellogenic, starved, or JH-deficient female adults increased Vg mRNA and protein levels, thereby implicating the pivotal role for insulin-like peptide signaling in the regulation of Vg gene expression and possible cross-talk between JH and insulin-like peptide signaling pathways. Reduction in JH synthesis or its action by RNAi-mediated silencing of genes coding for acid methyltransferase or methoprene-tolerant decreased expression of genes coding for insulin-like peptides (ILPs) and influenced FOXO subcellular localization, resulting in the down-regulation of Vg gene expression. Furthermore, JH application to previtellogenic female beetles induced the expression of genes coding for ILP2 and ILP3, and induced Vg gene expression. FOXO protein expressed in baculovirus system binds to FOXO response element present in the Vg gene promoter. These data suggest that JH functions through insulin-like peptide signaling pathway to regulate Vg gene expression.

Juvenile hormone (JH)² regulates many aspects of insect life including development and reproduction (1, 2). As a gonadotropic hormone, JH regulates oocyte maturation and vitellogenin (a protein synthesized by female insects and other animals and used to produce yolk, Vg) synthesis in the fat body and its

uptake by the developing oocytes (1). In locust (*Locusta migratoria*), injection of methoprene (JH analog) induced Vg gene expression in JH-deficient female adults (3). Similarly, in cockroach (*Blattella germanica*), treatment of JH rescued the JH-deficient female adults to express Vg in the fat body (4), and furthermore in this insect JH induced Vg mRNA in the isolated fat body *in vitro* (5). In addition to regulation of Vg synthesis, JH also enhances Vg uptake and promotes oocyte maturation in the ovary of the fruit fly, *Drosophila melanogaster* and other insects (1, 6). Although the gonadotropic role of JH has been clearly demonstrated in many insects, very little is known on the molecular mechanisms of JH action in regulation of reproduction.

The insulin-like peptide/TOR pathways sense nutrient status and play important roles in determining the tradeoff between survival and reproduction. In *D. melanogaster*, the mutant of insulin receptor (InR) yielded dwarf females with extended lifespan. These insects exhibited decreased JH levels and undeveloped ovarioles that resembled the ovarioles of wild-type flies that are in diapause, a delay in development in response to adverse environmental conditions (7). An exogenous application of JH analog methoprene to InR mutant dwarf females initiated vitellogenesis and restored lifespan similar to that of wild-type flies (7). Insulin-like peptide signaling and its downstream fork head transcription factor (FOXO) also mediate reproductive diapause response in the mosquito, *Culex pipiens* (8). In nematode, *Caenorhabditis elegans*, as well as in mouse, the insulin pathway plays an important role in the regulation of reproduction (9–11). The TOR pathway has been shown to link the elevated hemolymph amino acid levels from the blood meal to the expression of Vg gene in mosquito, *Aedes aegypti* (12–14). The *Ae. aegypti* larvae reared in inadequate nutrient conditions developed into smaller adults and showed an inactivated TOR pathway, lower JH levels, and needed additional blood meals to complete gonadotropic cycle (15). Application of JH III to these small mosquitoes could initiate vitellogenesis with only one blood meal (15). Recent work in *B. germanica* also showed that TOR mediated nutrition status affects Vg gene expression and JH levels (16). In lubber grasshoppers, a cumulative feeding threshold is required for vitellogenesis and can be

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¹ To whom correspondence should be addressed. Tel.: 859-257-4962; Fax: 859-323-1120; E-mail: rpalli@uky.edu.

² The abbreviations used are: JH, juvenile hormone; InR, insulin receptor; 20E, 20 hydroxyecdysone; Vg, vitellogenin; PAE post adult emergence; FHR, Forkhead response element; JHAMT, JH acid methyl transferase; Met, methoprene-tolerant; qRT, quantitative real-time; ILP, insulin-like peptide.

TABLE 1**Primers used to prepare dsRNA and in qRT-PCR**

T7 promoter sequence was added at the 5' end of each primer for amplification of template DNA used to prepare dsRNA. F, forward; R, reverse.

Name	Sequence
dsRNA	
ILP1-F	TCGCCTATAACGAATACGACGAGT
ILP1-R	TTTCCGGCAACATTCTGTCACAA
ILP2-F	AAGTCAGACATTACGAGATGGGTGC
ILP2-R	CGCCGCAATATTGGGACAGTTCTT
ILP3-F	CGGCAAAAGTAGTGGACGAATGTT
ILP3-R	CATTTCGAATGCCCATGGTTTGG
ILP4-F	AGATCACAATCCGACTGGGAGGAA
ILP4-R	CGTCCGTGTTAGTGATGTAATTATGTAGC
FOXO-F	AGCCGTCTCCCGACCCCTTAAATA
FOXO-R	TGCTGGTGATCCCTGAAATCGTCT
AKT-F	AAAAGGACGAAGTCGCTCAC
AKT-R	TCCGTATCGCTAGTAACCTG
qRT-PCR	
ILP1-F	TTACGTCTGGTCTTCACCGCACAT
ILP1-R	TGGTGGGTTTGGATTCCGGAGAGT
ILP2-F	TGGCCGAATACACACTTGTAGGA
ILP2-R	TCTTCTCCGCGAGTAGACCGCTTT
ILP3-F	AAAGTCTGCTTCACTTGCCTCCTC
ILP3-R	AATAGCGCACAGTTTCGGTGAGAGT
ILP4-F	TCCGAGAAGCGACCAAGCTACAA
ILP4-R	TCTGAAGTGATAGAAGCGCCAGCA
FOXO-F	CAACGAAGAGGGCAACAAGT
FOXO-R	CGCACTGATTTTCTGGTTT
AKT-F	CGACTTCACCAAGTGCAAAA
AKT-R	GCCCCCTCATTGTAAACGTA

obviated with JH treatment (17). Taken together, these studies suggest that vitellogenesis in insects is regulated by the nutrient-sensing insulin-like peptide/TOR pathways, but the cross-talk between JH and nutrient signals involved in regulation of vitellogenesis remains unclear. To address this long-standing question, we used the red flour beetle, *Tribolium castaneum*, as our model system because of the availability of whole genome sequence and well functioning systemic RNAi (18).

Previous studies from our laboratory showed that nutrition, insulin-like peptide, 20 hydroxyecdysone (20E), and JH regulate female reproduction in *T. castaneum*. 20E regulates oocyte maturation, JH regulates Vg synthesis in the fat body, and the nutritional signals mediated by insulin-like peptide/TOR pathways play key role in both Vg synthesis and oocyte maturation (19–21). In this study we show that both nutrition and JH are necessary for vitellogenesis, and both these signals work through the insulin-like peptide pathway and the downstream FOXO transcription factor to regulate Vg synthesis.

EXPERIMENTAL PROCEDURES

Beetles—Strain GA-1 of *T. castaneum* was maintained as described previously (19–21). Newly emerged female adults with untanned cuticle were kept separately and staged thereafter.

RNA Interference (RNAi) Assays—Gene-specific primers (reported in Refs. 19–21 or shown in Table 1) containing the T7 promoter sequence at their 5' ends were used to amplify 300–500-bp fragments from *T. castaneum* cDNA. Purified PCR products were transcribed to synthesize double-stranded RNA (dsRNA) using the MEGAscript T7 kit (Ambion, Austin, TX). The control dsRNA was prepared using a fragment of *Escherichia coli* male gene. Newly emerged female adults (~6 h post adult emergence (PAE)) or 3-day-old female pupae (appearance of black eyes but not black wings) were anesthetized with ether

vapor for 8 min. dsRNAs (400 ng/insect) were injected into beetles on the ventral side of the first abdominal segment using a aspirator tube assembly (Sigma) fitted with 3.5-inch glass capillary tube (Drummond) pulled by a needle puller (Model P-2000, Sutter Instrument Co.). Injected insects were allowed to recover for 8 h at room temperature (~22 °C) and then transferred to standard conditions. Knockdown efficiency of gene expression in the RNAi insects was calculated as the ratio of gene expression between target dsRNA-injected and control dsRNA-injected beetles.

Antibodies and Western Blots—Polyclonal antibodies made against *D. melanogaster* phospho-AKT (Ser-505), β -actin, and phospho-FOXO1 (Ser-256) were purchased from Cell Signaling Technology, and *Drosophila*-FOXO antibody was gifted from Dr. Marc Tatar, Brown University. *Tribolium*-Vg antibody was produced in our laboratory; a 1-kb fragment of Vg gene was cloned into pGEX-5X-1 expression vector (GE Healthcare), and the construct DNA was transformed into *E. coli* BL21 (DE3) cells (Invitrogen) to produce GST-Vg fusion protein. GST-Vg fusion protein was isolated by cutting a 50-kDa band from SDS-PAGE gel and injected into rabbit. After three injections, antiserum was collected and tested using isolated GST-Vg protein and fat body samples from various adult stages.

Isolated fat bodies were homogenized in PBS supplemented with protease inhibitor mixture (Sigma), boiled 5 min in SDS loading buffer, and centrifuged (12,000 \times g, 4 °C, 10 min) to collect the supernatant. Cytoplasm and nuclear extracts were also prepared from isolated fat bodies using a kit from Marligen Bioscience. 30 μ g of denatured protein samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in blocking solution (1 \times TBS with 5% nonfat milk and 0.02% Tween 20) and incubated overnight at 4 °C with 1:1000 primary antibody in blocking solution. Thermo Scientific Pierce ECL Western blotting substrate kit (containing goat anti-rabbit IgG conjugate with horseradish peroxidase) was used to visualize horseradish peroxidase.

Starvation, RNAi of JH Acid Methyl Transferase (JHAMT), Hormone Treatments and Fat Body Culture—To decipher the effect of nutrition and JH on vitellogenesis simultaneously, food deprivation plus RNAi of JHAMT experiments were performed by keeping JHAMT dsRNA-injected (male dsRNA as a control) female adults without food (with food as the control) and collecting samples every day during days 1–5 PAE for Vg expression analysis. After 4 days of starvation, JHAMT dsRNA-injected (male dsRNA as a control) beetles were fed on a hydrophene plus diet (50 μ g of hydrophene in 200 μ l of acetone/g diet, 200 μ l of acetone/g diet for the control) for 6, 12, and 24 h, and Vg2 mRNA levels were quantified. JH III was also applied topically (10 mM in acetone, 200 nl/insect) on JHAMT dsRNA-injected or previtellogenic beetles. Bovine insulin (10 mg/ml, 100 nl/insect, Sigma) was injected into female adults on days 1 and 2 PAE. Insulin-injected beetles were recovered by keeping them at room temperature for 8 h and transferred to standard conditions until use. Fat bodies were isolated from day 1 PAE female beetles and cultured in Schneider's *Drosophila* medium supplemented with 7% FBS. Dissected fat bodies were

washed in the medium three times and precultured in the medium for 1 h at 28 °C. JH (10 μ M) was added in the culture, and acetone was used as a control.

Quantitative Real-time Reverse Transcriptase PCR (qRT-PCR)—Total RNA was extracted from fat bodies isolated from 4 adults, 12 heads or 30 brains of staged, dsRNA-injected, starved, or hormone-treated female beetles using TRI reagent (Molecular Research Center Inc., Cincinnati OH). cDNA synthesis and qRT-PCR reactions were performed using the gene-specific primers (reported in Refs. 19–21 or shown in Table 1) and methods described previously (19, 20). Ribosomal protein gene rp49 was used as an internal control in qPCR analysis. The mean \pm S.D. of at least three independent replicates is shown.

Electrophoretic Mobility Shift Assays—Full-length FOXO and Met proteins were expressed in the baculovirus system as described in our previous publication (22). 30-bp primers (forward, 5'-GGTGAAACAACACGTAAACAAAATAAGAG-3'; reverse, 5'-CTCTTATTTTTGTTTACGTGTTGTTTCA-CC-3') containing FOXO response element (FHRE) identified in the Vg promoter were end-labeled using T4 polynucleotide kinase and [γ -³²P]ATP (6000 Ci/mmol) and purified by passing through a Sephadex G50 column. Proteins were mixed in assay buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 4% glycerol, 0.05 μ g/ μ l poly[dI-dC], and 20 μ M single-stranded, nonspecific DNA) and incubated at room temperature for 20 min, then the labeled probe was added to the reaction mixture and incubated for an additional 20 min at room temperature. The components of the reactions were then resolved on an 8% nondenaturing polyacrylamide gel. The gel was fixed in 7% acetic acid, dried onto a Whatman filter paper and visualized by phosphorimaging. In competition experiments a 100 \times concentration of unlabeled FHRE or a 30-bp unrelated double-stranded oligonucleotide was included in the initial reaction mixture before adding the labeled probe.

RESULTS

Nutrition and JH Regulate Vitellogenin Synthesis in Female Adults—Vitellogenin is coded by two genes, Vg1 and Vg2, in *T. castaneum*. Preliminary studies showed that the expression profiles of both the Vg genes are identical, and therefore, only Vg2 mRNA levels were measured in the studies described here. The antibodies made against Vg protein recognize products of both Vg genes. As shown by the appearance of blue dye mixed with flour in the gut, the adult females start feeding at about 2.5 days (Fig. 1A). The Vg2 mRNA was detected on the 3rd day PAE and increased rapidly on days 4 and 5 PAE (Fig. 1B). The vitellogenin proteins were detected on day 4 PAE and increased on day 5 PAE (Fig. 1C). In addition, starvation prevented an increase in Vg2 mRNA and Vg protein levels on days 4 and 5 PAE (Fig. 1, B and C). These data suggest that nutrition signals are required for expression of Vg genes.

To determine whether or not JH is also required for Vg synthesis, expression of the gene coding for a key enzyme involved in JH biosynthesis, JHAMT was knocked down by injecting JHAMT dsRNA. As shown in Fig. 1D, the mRNA levels of JHAMT started to increase on day 2 PAE and reached the maximum levels by day 4 or 3 in fed and starved insects, respectively. JHAMT expression was successfully silenced by injecting

JHAMT dsRNA into newly emerged female adults (Fig. 1E). JHAMT RNAi beetles synthesized no Vg2 mRNA in starved beetles but showed trace levels of Vg2 mRNA in fed insects (Fig. 1B). Similarly, JHAMT RNAi beetles showed no detectable Vg protein on days 4 and 5 (Fig. 1C). These data suggest that JH signaling is required for Vg synthesis. Feeding the starved beetles induced Vg2 mRNA by 12 h after feeding (Fig. 1F) and induced Vg protein levels by 24 h after feeding (Fig. 1G). However, this induction of Vg2 mRNA or protein levels by feeding was not observed in JHAMT RNAi beetles (Fig. 1, F and G). Feeding the diet plus hydroprene (JH analog) to starved JHAMT RNAi beetles increased the Vg2 mRNA and Vg protein levels by 24 h after feeding (Fig. 1, F and G). These data suggest that both nutrition and JH are required for Vg synthesis.

Insulin-like Peptides (ILPs) Control Vg Synthesis—*T. castaneum* genome contains four genes coding for insulin-like peptides (23). All four peptides contain both A and B chains as well as cysteines and other amino acids residues conserved among insulin-like peptides (Fig. 2A). The ILP1 coded by TC08479 is highly expressed in the brain, and the mRNA levels increase from day 0 to day 3 PAE (Fig. 2B). The ILP2 coded by TC07035 and ILP3 coded by TC00934 are expressed highly both in the brain and fat body. Although the ILP2 mRNA levels increased significantly on day 2 and day 3 PAE in both the brain and the fat body, the mRNA levels of ILP3 increased in the fat body on day 3 PAE (Fig. 2B). It is interesting that ILP2 mRNA levels began to increase on day 2 before feeding, and the ILP3 levels began to increase on day 3 after initiation of feeding. The ILP4 coded by TC00643 is expressed both in the brain and the fat body although at a lower level when compared with the levels observed for the other three ILPs (Fig. 2B).

Injection of bovine insulin into beetles on the 2nd day PAE induced Vg gene expression (Fig. 2C), and insulin induction of Vg gene expression was dose- and time-dependent (Fig. 2, C and D). Injection of insulin also induced Vg gene expression in starved beetles (Fig. 2E). However about 48-h exposure to insulin is required to reach the maximum levels of induction. Furthermore, injection of insulin into day-1 but not day-0 PAE beetles caused an increase in Vg mRNA levels (Fig. 2F). These data suggest activation of insulin pathway is sufficient to induce Vg gene expression.

To determine whether any of the four ILPs are required for Vg synthesis, ILP1, ILP2, ILP3, or ILP4 dsRNA was injected into the pupae, and the ILP1, ILP2, ILP3, ILP4, and Vg2 mRNA levels were quantified in adults on 3rd day PAE. As shown in Fig. 2G, injection of ILP2 and ILP4 dsRNAs reduced mRNA levels of only ILP2 and ILP4, respectively. Injection of ILP2 or ILP4 dsRNA did not affect the expression of genes coding for other three ILPs (Fig. 2G). However, injection of ILP1 dsRNA caused a reduction in the mRNA levels of both ILP1 and ILP3. Similarly, injection of ILP3 dsRNA caused a reduction in the mRNA levels of both ILP3 and ILP1 (Fig. 2G). The similarity between DNA sequences used to prepare ILP1 and ILP3 dsRNA was not very high, and there was no 22-nucleotide stretch that was identical between these two dsRNAs. Therefore, the reduction in the ILP3 mRNA in ILP1 RNAi insects and ILP1 mRNA in the ILP3 RNAi insects suggests that these two ILPs may regulate each other's expression. Interestingly, the Vg2 mRNA lev-

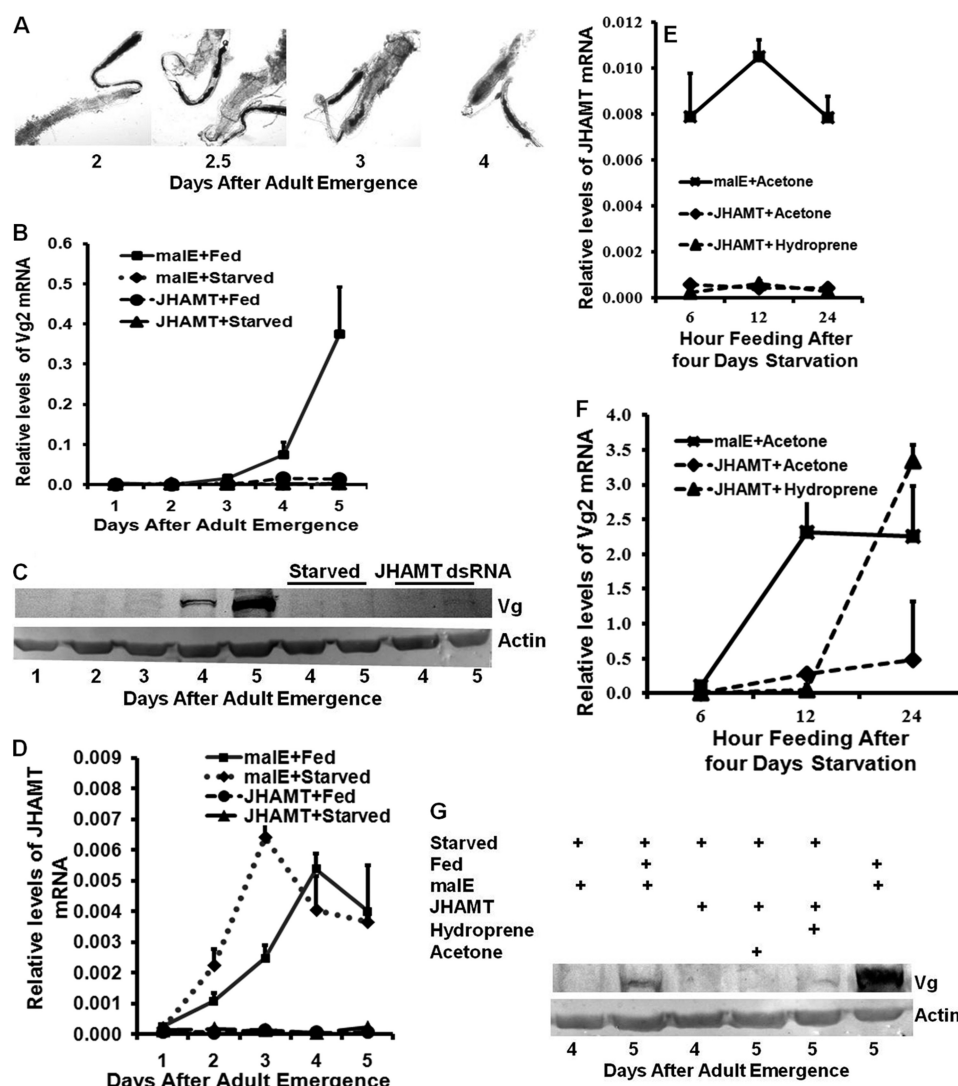


FIGURE 1. Nutrition and JH regulate Vg synthesis. *A*, adult female beetles start feeding beginning at 2.5 days PAE. Newly emerged female beetles were kept in a cup containing flour and yeast mixed with blue dye, and the alimentary canals were dissected and observed under a microscope at 2, 2.5, 3, and 4 days PAE. *B*, vitellogenin mRNA levels begin to increase on day 3 PAE, and JH is required for Vg gene expression. Total RNA was isolated from fat body dissected from normal adults injected with control maleE dsRNA and placed in a cup containing flour and yeast (*maleE + Fed*) or starved (*maleE + Starved*) or from those injected with JHAMT dsRNA and placed in a cup containing flour and yeast (*JHAMT + Fed*) or starved (*JHAMT + Starved*). The RNA was converted to cDNA, and the relative Vg2 mRNA levels were determined by qRT-PCR using RP49 as a control. The data shown are the mean \pm S.D. ($n = 3$). *C*, vitellogenin proteins begin to increase on day 3 PAE, and JH is required for Vg synthesis. Vitellogenin protein levels were detected by Western blots in proteins collected from adults females that were fed on flour and yeast from 1–5 days PAE, or starved beetles or JHAMT dsRNA-injected beetles collected on the 4th and 5th day PAE. Either Vg antibody (top panel) or β -actin (bottom panel) was used to detect respective proteins. *D*, JHAMT mRNA levels increase after adult emergence and reach the maximum levels by 3–4 days PAE. Relative levels of JHAMT mRNA were quantified in maleE + Fed, maleE + Starved, JHAMT + Fed, and JHAMT + Starved beetles using the total RNA isolated from adult females as described in legend for Fig. 1*B*. *E*, knockdown in the expression of gene coding for JHAMT by injection of JHAMT dsRNA is shown. JHAMT or maleE dsRNA was injected into newly emerged adult females, and the beetles were placed on a diet containing acetone or hydroprene (JH analog, 50 μ g of hydroprene in 200 μ l of acetone/g diet) for 6, 12, or 24 h. Expression of JHAMT mRNA levels were quantified, and the mean \pm S.D. ($n = 3$) is shown. *F*, JH is required for feeding induced Vg2 mRNA synthesis. Vg2 mRNA levels were quantified using total RNA isolated from beetles injected with maleE or JHAMT dsRNA and fed on a diet containing acetone or hydroprene for 6, 12, or 24 h as described in legend for Fig. 1*E*. *G*, JH is required for feeding induced Vg2 protein synthesis. Vitellogenin protein levels were monitored using Western blots, and proteins isolated from beetles injected with maleE or JHAMT dsRNA and fed on a diet containing acetone or hydroprene for 24 h as described in legend for Fig. 1*E*.

els were reduced by more than 90% in ILP1 or ILP3 dsRNA-injected insects when compared with its expression in control beetle-injected maleE dsRNA (Fig. 2*H*). The ILP2 and ILP4 dsRNA-injected insects showed about a 68 and 50% decrease in Vg2 mRNA levels, respectively. These data suggest that expression of genes coding for ILP2 and ILP3 is required for Vg gene expression. The ILP1 effect may be indirect due to regulation of its expression by ILP3. The ILP4 gene is expressed at very low levels in these tissues; therefore, it may not have a significant effect on Vg synthesis. Feeding starved beetles resulted in an

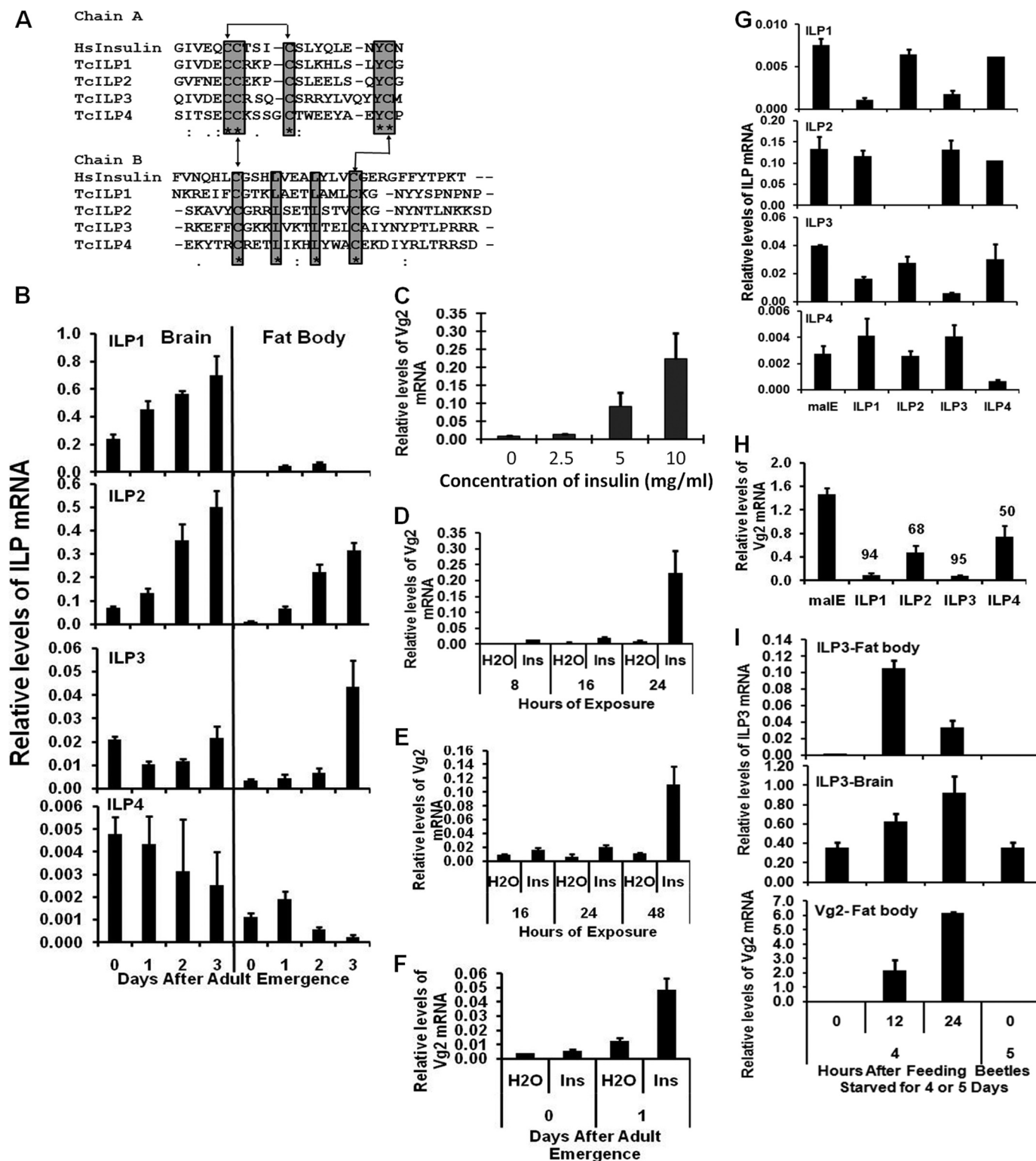
increase in both ILP3 and Vg2 mRNA levels (Fig. 2*I*). These data and expression profile of ILP3 (Fig. 2*B*) suggest that feeding induces expression of Vg gene through ILP3.

FOXO Mediates Nutrition Signals That Regulate Vg Synthesis—To further study the role of insulin-like peptide signaling in Vg synthesis, the gene coding for FOXO, a transcription factor involved in insulin-like peptide signaling was knocked down by RNAi. The mRNA levels of FOXO were decreased by more than 90% by 2 days after injection of dsRNA, indicating that FOXO gene was successfully silenced by RNAi (Fig. 3*A*). RNAi-

JH Works through ILP in Regulation of Vg Synthesis

mediated silencing of FOXO resulted in a precocious expression of Vg2 gene on day 2 PAE (Fig. 3B). The Vg2 mRNA levels in FOXO RNAi beetles on day 2 PAE are similar to those present in untreated day 5 PAE beetles (Fig. 3B). Similarly, silencing of FOXO caused precocious appearance of Vg protein on day 2 PAE (Fig. 3C). Interestingly, silencing of FOXO precociously induced Vg2 gene expression even in starved beetles. When

compared with the Vg mRNA levels in the control beetles injected with malE dsRNA, an increase in the Vg2 mRNA levels was detected in FOXO RNAi beetles that were starved from day 1 to day 4 PAE (Fig. 3D). These data suggest that FOXO suppresses the expression of Vg genes in adults after their emergence until nutritional signals are received via insulin-like peptide/TOR pathway when beetles began to feed.



We determined the levels of FOXO protein in the whole cell as well as in the nuclear and cytoplasmic fractions isolated from the fat body dissected from beetles between day 2 (previtellogenic, before initiation of feeding) and day 5 (vitellogenic, after initiation of feeding) PAE. FOXO protein levels in the whole cell increased gradually from day 2 to day 5 PAE (Fig. 3E). Phosphorylated FOXO protein detected by phosphospecific antibody showed an increase on day 3 and remained high on day 4 but decreased on day 5 PAE (Fig. 3E). FOXO protein levels gradually increased in the cytoplasm from day 2 to day 5 PAE. In contrast, FOXO protein levels decreased in the nuclear fraction from day 2 to day 5 PAE (Fig. 3E). These data suggest that dephosphorylated FOXO resides in the nucleus and likely binds to the Vg gene promoters to prevent its expression until insulin-like peptide/TOR pathways are turned on by nutrition. When these pathways are turned on by nutrition, the FOXO protein is phosphorylated and transported out of the nucleus, allowing expression of Vg genes.

AKT Transduces Nutrition Signals That Regulate Vg Synthesis—AKT is another important protein involved in insulin-like peptide signaling. Insulin-like peptide induces the phosphorylation of this protein, and AKT in turn influences FOXO phosphorylation and localization. Phosphorylated AKT levels are lower on day 1 PAE but increased to reach the maximum levels by day 3 PAE when female beetles begin to ingest food and synthesize Vg (Fig. 3F). Similarly, phosphorylated AKT levels were lower in starved beetles but increased by 12 h after initiation of feeding and reached the maximum levels by 24 h after feeding (Fig. 3G). These data suggest that nutritional signals enhance phosphorylation of AKT. To further confirm the key role of AKT in the nutrition-induced Vg synthesis, AKT was knocked down by RNAi. Injection of AKT dsRNA into adult females resulted in more than a 90% reduction in AKT mRNA levels in day 4 PAE adults (Fig. 3H). Knockdown in the expression of gene coding for AKT resulted in a reduction in Vg synthesis when compared to Vg protein levels in beetles injected with malE dsRNA (Fig. 3I). Feeding of starved beetles induces Vg synthesis as shown in malE dsRNA-injected insects (Fig. 3I). However, in the AKT RNAi-starved female adults, feeding failed to induce Vg synthesis, suggesting that AKT is required for transducing nutrition signals that regulate Vg synthesis (Fig. 3I).

Juvenile Hormone Works through the Insulin-like Peptide Signaling Pathway to Regulate Vitellogenesis—The methoprene-tolerant (Met), a bHLH-PAS family transcription factor plays an important role in JH action. To determine the role of JH in

Vg gene expression, we injected JHAMT, Met, or FOXO dsRNA into newly emerged adults, and monitored the mRNA levels of these genes in RNAi beetles. More than a 80% decrease in JHAMT, Met, and FOXO mRNA levels in RNAi beetles when compared with the levels in the control beetles was observed on day 3 after injection (Fig. 4A). Knockdown in the expression of JHAMT or Met resulted in a decrease in the levels of Vg protein (Fig. 4B). The decrease in Vg protein levels in Met or JHAMT RNAi beetles is not due to the lack of feeding as RNAi animals showed blue dye in their alimentary canal (Fig. 4C). Injection of JHAMT or Met dsRNA into adult female beetles on day 0 decreased Vg2 mRNA levels detected on day 4 PAE (Fig. 4D). In addition, injection of bovine insulin into JHAMT or Met RNAi beetles rescued Vg2 mRNA levels similar to those detected in control beetles injected with malE dsRNA (Fig. 4D). Also, knockdown in the expression of FOXO in JHAMT or Met RNAi beetles increased Vg2 mRNA levels (Fig. 4E). Interestingly, the levels of FOXO in the cytoplasm extract, phosphorylated FOXO, and Vg decreased in both JHAMT and Met RNAi beetles when compared with their levels in control beetles (Fig. 4F). Application of the JH analog, hydroprene, or JH III rescued both FOXO and Vg levels in JHAMT RNAi beetles. The application of JH on day 1 PAE beetles induced precocious Vg2 gene expression (Fig. 4F). In addition, knockdown in the expression of genes coding for insulin receptor or AKT prevented JH-induced expression of Vg gene (Fig. 4G). These data suggest that JH regulates Vg gene expression by working through insulin-like peptide signaling pathway affecting FOXO localization in the fat body cells.

To determine whether the JH influence on insulin-like peptide pathway is through regulation of synthesis of ILPs, the mRNA levels of ILPs were quantified in the fat body and brain dissected from adult female beetles injected with control malE, JHAMT, or Met dsRNA. Knockdown in the expression of genes coding for JHAMT or MET caused a decrease in mRNA levels of ILP2 and ILP3 in both fat body and brain (Fig. 5A). In addition, application of JH III to JHAMT RNAi beetles rescued the decrease in ILP2 and ILP3 mRNA levels in these insects (Fig. 5, B and C). Neither JHAMT nor Met RNAi affected ILP1 or ILP4 expression (Fig. 5A). Culturing fat body dissected from day 1 PAE beetles in the medium containing JH III for 5 and 10–12 h induced ILP2 but not ILP1, ILP3, or ILP4 mRNA (Fig. 5D). Similarly, topical application of JH III to day 1 PAE adults induced ILP2 but not other ILP mRNA in the brain (Fig. 5E). Exposure of previtellogenic day-1 PAE beetles to JH induced ILP2 mRNA

FIGURE 2. Insulin-like peptides regulate Vg gene expression. A, shown is a comparison of amino acid sequences of four ILPs with the amino acid sequence of human insulin chains A and B. Cysteines and other amino acids conserved in all four ILPs are highlighted. *, identical amino acids in all five proteins; and ., conserved amino acids. HS, *Homo sapiens*; Tc, *T. castaneum*. B, expression patterns of ILP1 (TC08479), ILP2 (TC07035), ILP3 (TC00934), and ILP4 (TC00643) in the fat body and brain in day 0–3 PAE female beetles were determined by qRT-PCR. C, bovine insulin induces Vg synthesis. Bovine insulin was injected into female adults on day 2 PAE (100 nl of 0, 1.25, 2.5, and 10 mg/ml), and 24 h later Vg2 mRNA levels in the fat body were determined by qRT-PCR. D, shown is the time-course of Vg2 mRNA induction by insulin (Ins). Vg2 mRNA levels were measured by qRT-PCR in the fat body isolated from day 2 PAE beetles at 8, 16, and 24 h after injection of 100 nl of H₂O or 10 mg/ml bovine insulin. E, insulin induces Vg2 mRNA even in starved beetles. Day 2 PAE female beetles were injected with bovine insulin and starved for 16, 24, and 48 h after injection, and Vg2 expression in the fat body was measured by qRT-PCR. F, insulin induced Vg2 mRNA even in day 1 beetles before initiation of feeding. Bovine insulin was injected into day 1 PAE female beetles, and 24 h later Vg2 mRNA levels in the fat body were measured by qRT-PCR. G, injection of ILP dsRNA reduced respective ILP mRNA for ILP2 and ILP4. Control malE or ILP1, ILP2, ILP3, or ILP4 dsRNA was injected into day 3 pupa, and ILP mRNA levels were quantified in day 3 adults. H, ILPs are required for Vg synthesis. ILP dsRNAs were injected into pupae, and the total RNA isolated from the fat body dissected on day 4 PAE was used to quantify Vg2 mRNA levels. The numbers on the top of bars show percent reduction in Vg mRNA levels compared to control. I, feeding induced ILP3 and Vg2 mRNA levels. Female adult beetles starved for 4 days were fed on diet for 0, 12, and 24 h or starved for an additional 24 h. The total RNA was isolated from dissected fat body or brain, and ILP3 and Vg2 mRNA levels were determined.

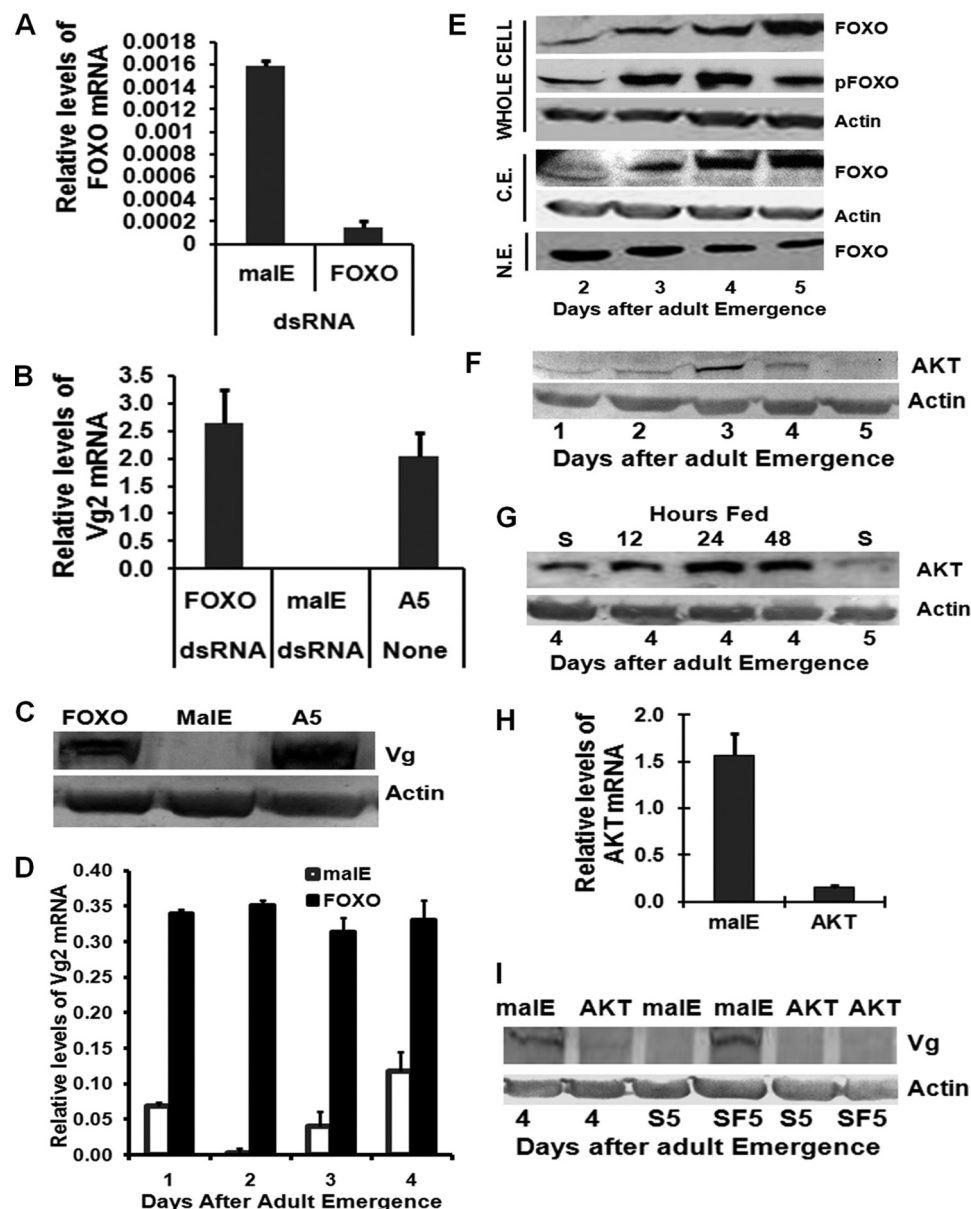


FIGURE 3. Vitellogenin gene expression is regulated by insulin-like peptide functioning through FOXO. *A*, shown is knockdown efficiency of gene coding for FOXO after injection of FOXO dsRNA. Control male or FOXO dsRNA were injected into day 3 pupa, and FOXO mRNA levels were quantified in day 2 adults. *B*, precocious expression of Vg2 on day 2 PAE in FOXO dsRNA-injected female adults is shown. The Vg2 mRNA levels were quantified using total RNA isolated from fat body dissected from male or FOXO dsRNA-injected beetles on day 2 PAE. The Vg2 mRNA levels in day 5 control female adults are also shown. *C*, precocious appearance of Vg2 protein in FOXO dsRNA-injected female adults on day 2 PAE is shown. Proteins isolated from male and FOXO dsRNA-injected beetles were collected on day 2 PAE and analyzed by Western blots using Vg antibodies. The Vg levels in day 5 control female adults are also shown. *D*, vitellogenin mRNAs are detected in FOXO dsRNA-injected beetles that were starved for 1–4 days. FOXO or male dsRNA was injected soon after adult emergence, and the beetles were maintained without diet for 1–4 days. Total RNA was isolated, and Vg2 mRNA levels were quantified. *E*, both FOXO and phosphorylated FOXO levels increased from day 2 to day 5 PAE. Whole cell lysate, cytoplasm extract (C.E.) and nuclear extract (N.E.) were prepared from the fat bodies dissected from day 2–5 adults, and 30 μ g protein from each sample was analyzed on Western blots using FOXO, phosphorylated FOXO antibodies. *F*, phosphorylated AKT protein levels correlate with Vg gene expression. Shown is the developmental pattern of phospho-AKT (Ser-505) in the fat body of female adults during day 1–5 PAE. *G*, feeding increases phospho-AKT protein levels. Levels of phospho-AKT (Ser-505) in the fat body isolated from 4-day-starved female adults after a feeding for 0, 12, 24, and 48 h. The level of phospho-AKT in 5-day-starved female adults is also shown. *H*, knock down efficiency of gene coding for AKT after injection of AKT dsRNA is shown. Control male or AKT dsRNA were injected into day 3 pupa, and AKT mRNA levels were quantified in day 3 adults. *I*, AKT is required for Vg synthesis. AKT or male dsRNA was injected into day 0 PAE female adults. Vg protein levels were analyzed by Western blot in fat bodies isolated from day 4 PAE beetles (4). Vg protein levels were also analyzed in the fat bodies isolated from AKT RNAi or male RNAi 5-day-starved beetles (S5) and 5-day-starved and 24-h fed beetles (SF5).

levels in the fat body beginning at 6 h after application of JH III (Fig. 5E). The mRNA levels of the other three ILPs tested did not change after JH III application. Interestingly, in these beetles Vg2 mRNA levels were induced only after 36 h of exposure to JH III (Fig. 5F).

FOXO Regulates Vg Gene Expression by Binding to a DNA Response Element—The promoter region of Vg2 gene contains two DNA elements that match with consensus FOXO response element located at 984 (FHRE2) and 341 (FHRE1) nucleotides upstream from the transcription start site (Fig. 6A). Full-length

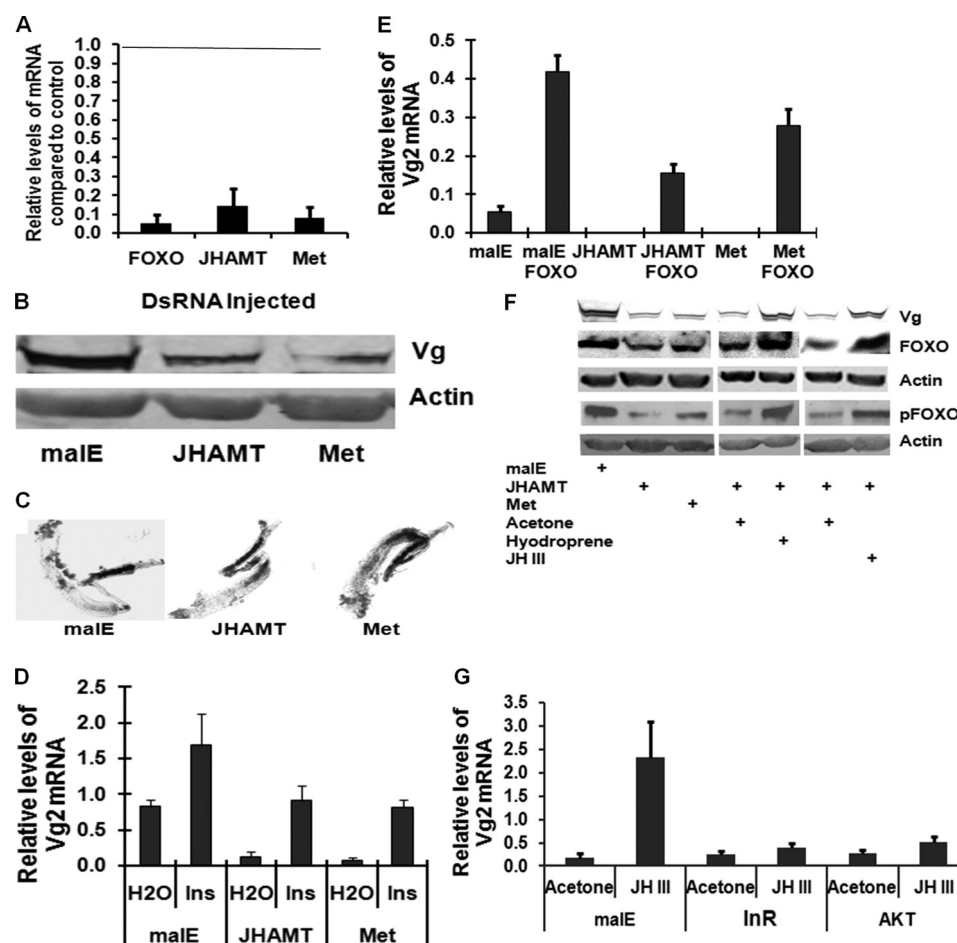


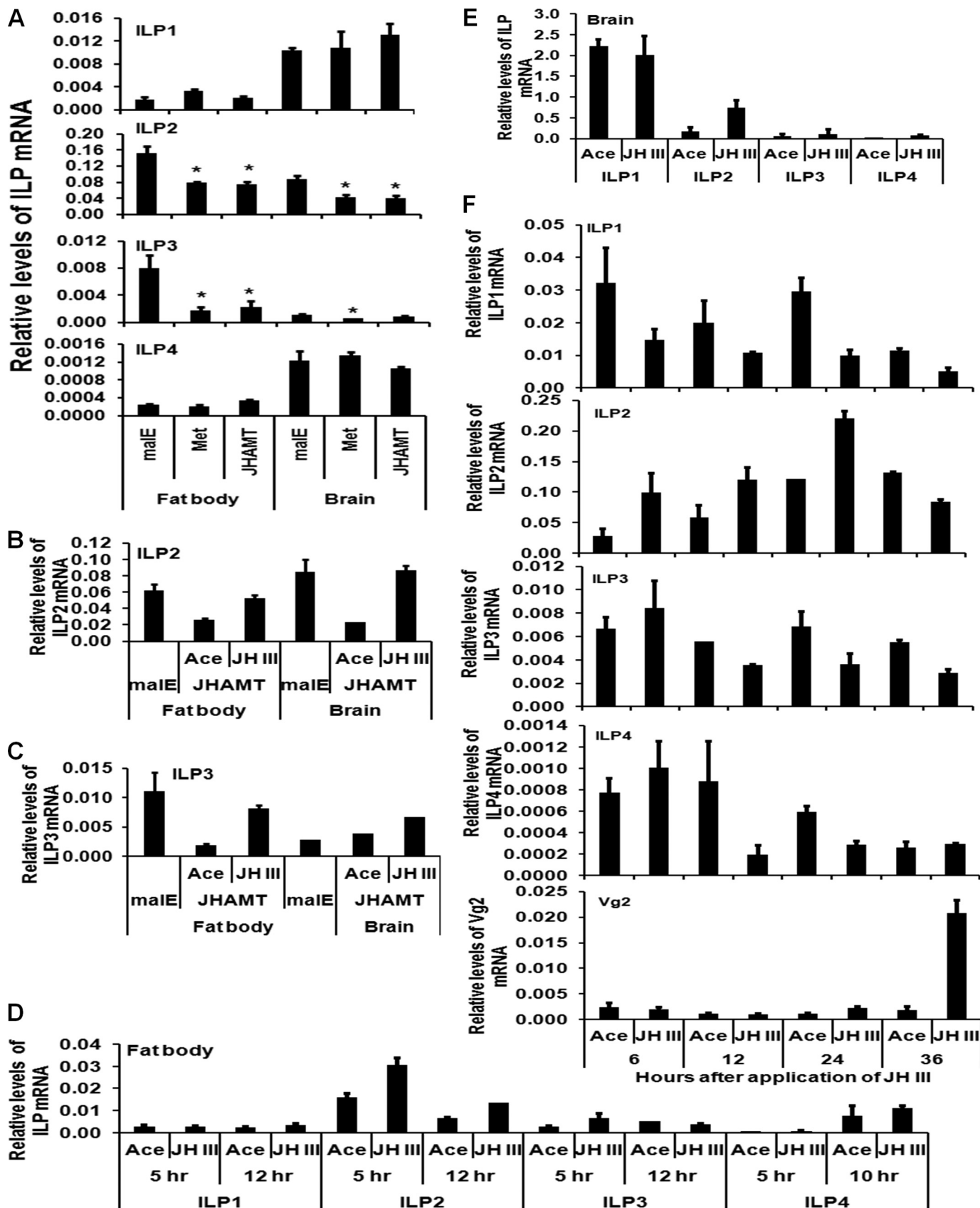
FIGURE 4. JH regulates Vg gene expression through the insulin-like peptide pathway. *A*, knockdown efficiency of genes coding for FOXO, JHAMT, and Met after injection of FOXO, JHAMT, and Met dsRNA is shown. Control malE or FOXO, JHAMT, or Met dsRNA were injected into newly emerged adults, and FOXO, JHAMT, and Met mRNA levels were quantified in day 3 adults. FOXO, JHAMT, and Met mRNA levels in dsRNA-injected beetles are shown as the ratio compared with control beetles (set as 1). *B*, knockdown in the expression of genes coding for JHAMT or Met results in reduced levels of Vg protein. Western blots were used to determine Vg protein levels in the fat body of female adults on day 4 PAE after injection of malE JHAMT or Met dsRNA soon after adult emergence. *C*, the reduction in the Vg levels in JHAMT or Met RNAi beetles is not due to starvation. Food ingestion showed by the existence of a blue color in the gut of female adults at day 4 PAE after injection of malE, JHAMT, or Met dsRNA. *D*, injection of bovine insulin (*Ins*) induces Vg2 mRNA levels even in JHAMT or MET RNAi beetles. mRNA levels of Vg2 in the fat body of female adults on day 4 PAE after injection of malE JHAMT or Met dsRNA into newly emerged adults. On day 2 PAE, bovine insulin (10 mg/ml, 100 nl per insect, H₂O as a control) was injected into dsRNA-injected beetles. *E*, precocious expression of Vg2 gene in the FOXO RNAi insects occurs even in JHAMT or Met RNAi beetles. Vg2 expression in the fat body of female adults on day 3 PAE after injection of malE, malE+FOXO, JHAMT, JHAMT+FOXO, Met, or Met+FOXO dsRNA is shown. A total volume of 100 nl containing 400 ng of each dsRNA per insect was injected into newly emerged female adults. *F*, JH regulates Vg synthesis by affecting FOXO protein phosphorylation. Protein levels of Vg2 and FOXO in the cytoplasm extract and phospho-FOXO in the whole cell extract are shown. The dsRNAs were injected into newly emerged females, and beginning on day 2 PAE, they were maintained on diet containing hydroxyphenyl or acetone or normal diet, but 1 μ l of 10 μ M JH III in acetone or acetone alone were topically applied. The proteins from fat body were collected on day 4 PAE and analyzed by Western blots using Vg, FOXO, and actin antibodies. *G*, both InR and AKT are required for JH induction of Vg gene expression. Control malE, InR, or AKT dsRNA were injected into newly emerged adults. 1 μ l of 10 μ M JH III in acetone or acetone alone was topically applied on day 2 PAE. Vg2 mRNA levels were quantified in the total RNA isolated from the fat body dissected at 24 h after application of JH.

Met and FOXO proteins expressed from *T. castaneum* cDNAs in baculovirus expression system (Fig. 6B) were used to determine whether either of these proteins bind to the two FHREs identified in the Vg promoter. Only FOXO, but not Met, bound to FHRE2 present in the Vg promoter (Fig. 6C). The binding of FOXO to FHRE2 is specific because the binding is competed by a specific but not by a nonspecific unlabeled probe (Fig. 6C). Neither FOXO nor Met bound to FHRE1 present in the Vg gene promoter. These data suggest that FOXO may regulate Vg gene expression by binding to FHRE2.

DISCUSSION

The major contribution of this study is the discovery that JH regulates Vg synthesis through the insulin-like peptide signal-

ing pathway. The data included in this paper provided answers to the long-standing question on JH regulation of Vg synthesis. JH regulation of female reproduction, especially Vg synthesis, is known for a long time, but the mechanism of action remained elusive (1). Exogenous JH application induces Vg production in the fat body of several insects (4, 5, 24–26). In most insects a longer exposure (up to 24 h) to JH or its analog methoprene is required to induce Vg gene expression. Only in the cockroach is induction of Vg mRNA by JH is fast, but in this case requirement of other protein factors for complete Vg synthesis has also been suggested (4). Taken together, these data suggest that synthesis of some new proteins that aid in Vg gene expression are required for JH induction of Vg gene expression. However, the identity of these proteins remained elusive for several years.



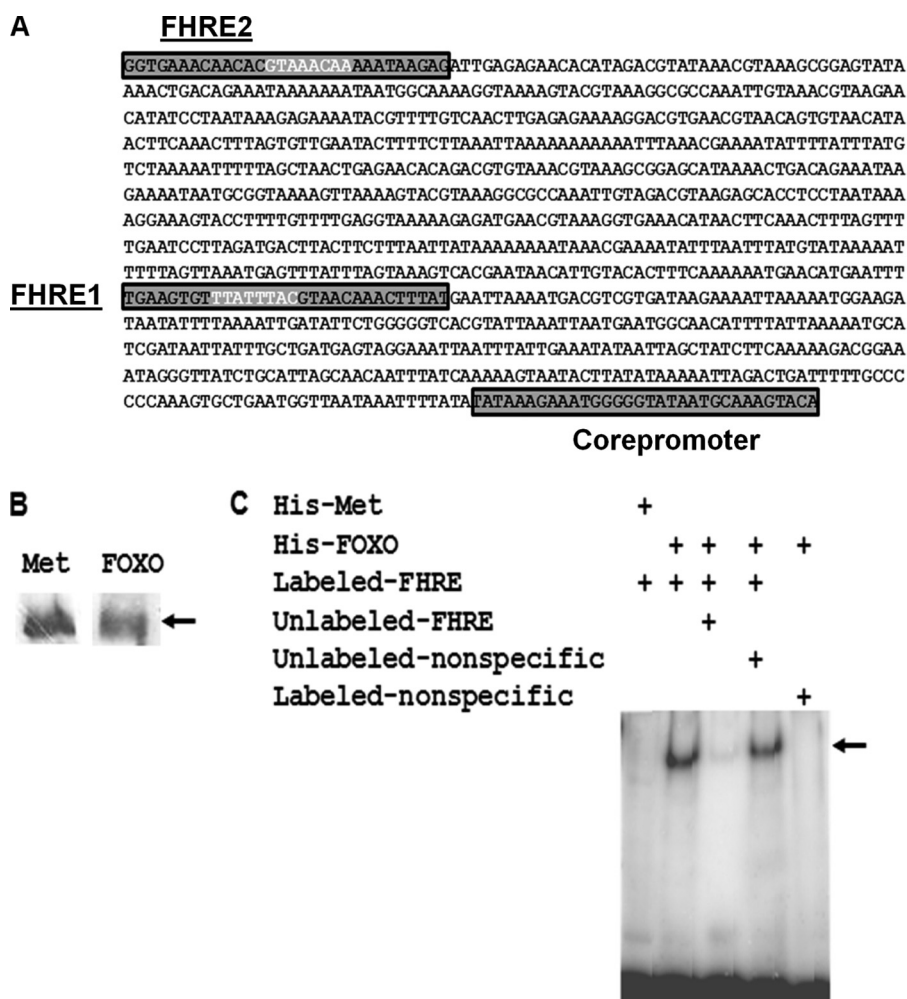


FIGURE 6. **FOXO protein binds to FHRE present in the Vg2 promoter.** A, shown is the sequence of Vg2 promoter. The core promoter and two FHRE identified are labeled. The sequences in the *dark background* and labeled as FHRE have been used in EMSA assay. B, FOXO and Met His-TcMet-V5 fusion protein expressed in insect cells using a baculovirus expression system were analyzed by a Western blot using antibodies made against V5 tag. C, FOXO protein binds to FHRE2 identified in the Vg2 promoter. FOXO and Met were incubated with labeled FHRE2. In competition studies, 100-fold excess of unlabeled FHRE or 100-fold excess of unlabeled unrelated double-stranded oligonucleotide of the same length was added. 30 bp of unrelated oligonucleotide were also labeled and used as a nonspecific probe. The products were separated on a 6% non-denaturing gel, and the signals were detected using phosphorimaging.

Previous studies in our laboratory showed that JH regulates Vg synthesis in *T. castaneum* (20). Exogenous application of either JH or insulin or dsRNA of FOXO induced precocious expression of Vg gene in previtellogenic female adults before initiation of feeding. Knockdown in the expression of genes coding for key players in JH biosynthesis (JHAMT), JH action (Met), or insulin-like peptide signaling (InR and AKT) caused a reduction in Vg mRNA levels, suggesting that both these signals are required for Vg gene expression. Interestingly, injection of insulin or FOXO dsRNA rescued Vg gene expression in

JHAMT and Met RNAi beetles. Also, RNAi-mediated silencing of InR or AKT blocked the JH-induced Vg2 gene expression, suggesting that JH likely regulates Vg gene expression by working through insulin-like peptide pathway. Previous studies showed that the application of JH initiates vitellogenesis in small mosquito adults developed from larvae raised under limited nutrition conditions (15) and also in grasshoppers that were raised at sub-threshold feeding conditions (17). In these insects JH likely overcame nutrition limits by enhancing insulin-like peptide signaling pathway activity. This hypothesis is

FIGURE 5. **Juvenile hormone regulates expression of genes coding for ILPs.** A, knockdown in the expression of gene coding for JHAMT or Met results in reduced ILP2 or ILP3 mRNA levels in the fat body and brain. The levels of ILP mRNAs in the fat body and brain on day 4 PAE in the female beetles injected with malE, JHAMT, or Met dsRNA and measured by qRT-PCR are shown. dsRNAs were injected into newly emerged female adults, and the total RNA isolated from fat body dissected on day 4 PAE was used to quantify ILP mRNA levels. B and C, application of JH III rescues reduction in ILP2 (B) and ILP3 (C) mRNA levels in JHAMT RNAi beetles. The levels of ILP2 (B) and ILP3 (C) mRNAs in the fat body and brain in day 3 PAE female beetles injected with malE, JHAMT, or Met dsRNA are shown. JH III or acetone (Ace) was topically applied to JHAMT dsRNA-injected female adults at day 2.5 PAE, the fat body was dissected at 12 h later, and the total RNA was isolated and used to quantify ILP2 or ILP3 mRNA levels. D, ILP2 mRNAs are induced by JH III in *in vitro* cultured fat body. Fat bodies were isolated from day 1 PAE female beetles and cultured in Schneider's *Drosophila* medium supplemented with 7% FBS and 1% antibiotics and 10 μ M JH III or acetone. After 5 and 10–12 h of culture, the fat bodies were collected, total RNA was isolated, and ILP2 mRNA levels were quantified. E, ILP2 mRNAs are induced in the brain by topically applied JH III. mRNA levels were quantified in the brain dissected from the day 1 PAE beetles treated (topical application) with JH III for 6 h. F, shown is sequential induction of ILP2 and Vg2 mRNA by JH. JH III was topically applied on the day 1 PAE female beetles every 12 h. The levels of ILPs and Vg2 mRNAs were analyzed in the fat bodies dissected from the beetles treated with JH III for 6, 12, 24, and 36 h.

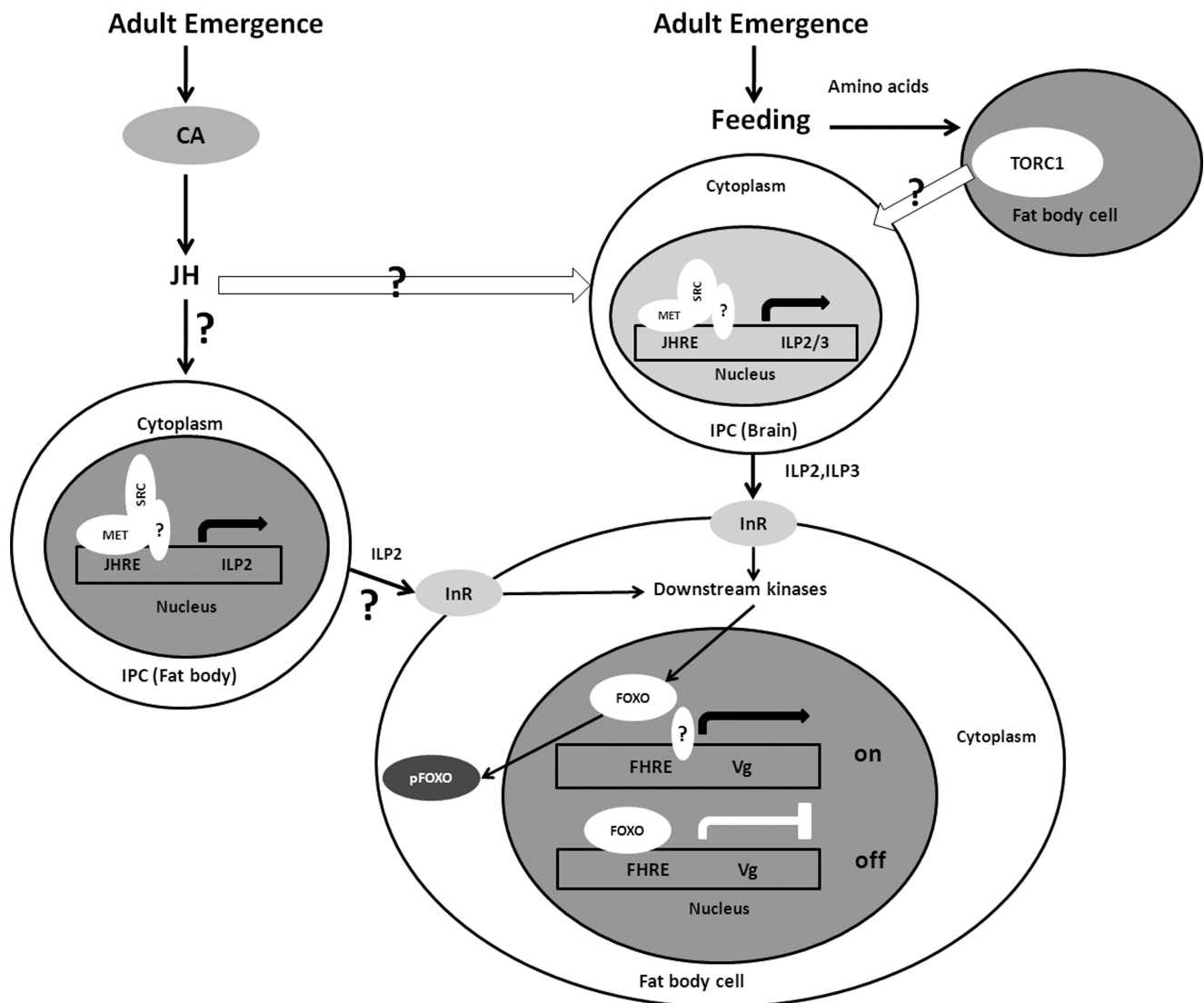


FIGURE 7. **A model for JH and ILP regulation of Vg synthesis.** After adult emergence, JH III titers increase and induce expression of genes coding for ILP2 and ILP3 in the fat body and the brain. The ILPs then work through the insulin-like peptide signaling pathway to phosphorylate FOXO, resulting in expression of Vg genes. Feeding increases amino acid concentration and turn on production of ILP3 through most likely the TOR pathway. The ILP3 then induces expression of Vg genes through insulin-like peptide signaling pathway and FOXO. Thus, both JH and feeding function through the insulin-like peptide signaling pathway and regulate expression of genes coding for Vg. *JHRE*, JH response element; *CA*, corpora allata; *FHRE*, Forkhead response element; *SRC*, steroid receptor co-activator; *TORC1*, target of rapamycin complex 1; *IPC*, insulin-like peptide producing cell.

supported by our data that showed a decrease in levels of phosphorylated FOXO and cytoplasmic FOXO in the fat body from JHAMT and Met RNAi insects and JH induction of ILP2 gene expression in both fat body and brain. In addition, the expression of ILP2 gene was down-regulated in the JHAMT and Met RNAi insects. Exogenous JH application to day 1 PAE female adults first induced the expression of ILP2 by 6 h after treatment then triggered Vg gene expression by 36 h after treatment. Taken together, these data conclusively showed that JH regulates Vg gene expression in the beetle, *T. castaneum*, by inducing the expression of ILP2. Interestingly, an increase in ILP mRNAs in methoprene-treated honey bees has been reported previously (27). Thus, there have been some hints in the previous studies that JH may induce ILPs, and our data included in this paper confirmed these previous observations and clearly showed that in *T. castaneum* JH regulates Vg synthesis by inducing expression of ILPs (Fig. 6). This could be a possible

mechanism of action in most insects where JH regulates Vg gene expression. However, direct studies in these insects need to be conducted to test this hypothesis.

Nutrient-sensing insulin-like peptide signaling/TOR pathways regulate vitellogenesis in fruit flies, mosquitoes, honey bees, and beetles (21, 27–29). Recent studies in *D. melanogaster* showed that secretion of ILPs is controlled by the nutritional status mediated by TOR signaling, suggesting a link between TOR and insulin pathways in transducing nutritional signals (30). Insulin pathway is known to function as a nutrient-sensing pathway coupling dietary conditions to the control of growth and energy metabolism (31). Under limited nutrient conditions, the inactivated insulin pathway contributes to the decrease in reproduction in the diapausing fruit flies (7, 32) mosquitoes (8) and during the dauer state of nematodes (33). In *T. castaneum* as reported in other insects (15–17), vitellogenesis is regulated by nutrient status as reproduction is one of the

primary energy consuming processes in beetle life. Feeding induces expression of ILP3 and turns on insulin-like peptide pathway, which in turn induces expression of Vg genes (Fig. 7). Knockdown in the expression of genes coding for ILP3 reduced Vg gene expression. Moreover, injection of bovine insulin induced Vg gene expression in previtellogenic female adults that have not initiated feeding as well as in starved beetles. RNAi-mediated silencing of genes such as InR, Chico, pi3k, and AKT coding for positive regulators of insulin-like peptide signaling pathway down-regulated Vg gene expression (21). In contrast, silencing of gene coding for FOXO induced Vg2 gene expression precociously in day 2 female adults. These data suggest that expression of Vg genes in *T. castaneum* is under the control of FOXO regulated by insulin-like peptide signaling pathway. Interestingly, in nematodes *C. elegans* silencing of gene coding for DAF-2 (InR homologue) and DAF-16 (FOXO homologue) showed similar effects as seen in *T. castaneum* (11), suggesting a conserved role for insulin-like peptide signaling pathway in regulation of vitellogenesis. FOXO RNAi induced expression of Vg gene in previtellogenic beetles that did not feed as well as in starved female beetles, suggesting that nutrition regulation of vitellogenesis is mediated by FOXO, which enters the nucleus and regulates target genes under limited nutrient conditions (33). In addition to insects (27–30, 34) and nematodes (11), the control of vitellogenin synthesis by insulin signaling has also been reported in frog (35) and fish (36), suggesting that insulin regulation of vitellogenesis is well conserved through evolution.

JH has been shown to be involved in the nutrient-mediated regulation of reproduction in many insect species (7, 8, 15, 16). The regulation of JH titer by nutrient signals working through the insulin-like peptide/TOR pathways was observed in fruit flies (7, 37), mosquitoes (15), and cockroaches (16). However, female sterility of an insulin/IGF-like signaling mutant chico of *D. melanogaster* is not mediated by alterations in JH levels (29). Our data showed that in *T. castaneum* the control of vitellogenesis by nutrition is mediated by insulin-like peptide pathway and does not appear to function through regulation of JH biosynthesis as starvation did not change the expression of gene coding for JHAMT, a key enzyme involved in JH biosynthesis, and its expression correlates very well with JH titer in this beetle and other insects (20, 38). Unlike in *D. melanogaster*, JH induces the expression of ILPs and influences the expression of Vg genes indirectly in *T. castaneum*.

JH and 20E as well as insulin-like peptides regulate insect development, metamorphosis, and reproduction; recent studies raise these three hormones as a sophisticated endocrinal network. In *D. melanogaster* larva, 20E signaling negatively controls growth rates (39) and induces programmed autophagy (40) by antagonizing the insulin/PI3K pathway. Insulin-like peptide-mediated activation of PI3K and ecdysone synthesis in the prothoracic glands, leading to initiation of metamorphosis in *D. melanogaster*, has also been observed (41). In *Manduca sexta*, JH is required to couple imaginal disc formation with nutrition (42). Here, JH may function through insulin-like peptide signaling pathway. The data presented here clearly showed that JH regulates the synthesis of insulin-like peptide that in turn regulates Vg synthesis, thus providing an answer to the

long-standing question on the mechanism of JH action in regulation of vitellogenesis. This study thus identified an additional function, regulation of insulin-like peptide synthesis, for pleiotrophic hormone, JH. Future studies on cross-talk among JH, 20E, and insulin-like peptide signaling pathways will provide explanations for physiological and developmental changes manifested in response to nutritional, environmental, and hormonal signals during insect development and reproduction.

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Zhentao Sheng, Jingjing Xu, Hua Bai, Fang Zhu and Subba R. Palli

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